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(54) Title: CROSS-REACTIVE ALLERGEN

(57) Abstract

Screening of a Kentucky Bluegrass pollen-λgt11 library with sera from patients allergic to grass pollen led to the identification of a partial cDNA clone, KBG51. Nucleotide sequence analysis of KBG51 indicated that the polypeptide encoded by this cDNA is different from that of the known recombinant grass pollen allergens. Using murine antiserum to a GST-KBG51 fusion protein, produced with aid of pGEX-2T-1 expression system, two polypeptides of about 30 and 59 KDa in size, were detectable in SDS-PAGE immunoblot analysis of KBG pollen proteins. The reactivity of this antiserum, with a number of polypeptides, which ranged in size from 29.5 to 115 kDa, from pollen extracts of several grasses, birch, ragweed and parietaria and the hybridization of RT-PCR products from various pollens with radiolabelled KBG51-cDNA, demonstrated the cross-reactivity (CR) of this AL with other pollen ALs. Because of the broad CR, the protein(s) corresponding to KBG51 has been designated as CRAL51. Analysis by ELISA using sera of about 1000 individuals worldwide who were allergic to pollens demonstrated that individuals from a variety of geographical areas possessed IgE antibodies that recognized the GST-KBG51 fusion protein. On the basis of these findings, CRAL51 represents a member of a family of highly cross-reactive ALs in plant pollens.

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TITLE OF THE INVENTION

CROSS-REACTIVE ALLERGEN

FIELD OF INVENTION

The present invention is related to the field of molecular genetics and is particularly concerned with the cloning of a cross-reactive allergen.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States patent application Serial No. 08/181,383 filed January 14, 1994.

BACKGROUND TO THE INVENTION

Pollen allergens are multideterminant proteins or glycoproteins capable of eliciting IgE-mediated allergic diseases such as hayfever and asthma in approximately 17% of the population who are genetically predisposed to develop allergies (ref. 1 - Throughout this specification, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the disclosure. The disclosure of these references are hereby incorporated by reference into the present disclosure). In contrast to some other allergens (e.g., cat or house dust mite allergens), the global distribution of pollens of a large variety of monocotyledonous (grasses) and dicotyledonous plants (trees and weeds) preclude any realistic possibilities that individuals allergic to pollen allergens can avoid these aero-allergens. The current treatment for hayfever consists primarily in symptomatic relief. Sufferers take drugs, such as anti-histamines and steroids, which do not suppress the formation of IgE antibodies and often have harmful side effects.

Attempts to downregulate the IgE immune responses of allergic subjects by the "time-honoured" immunotherapy consist of a series of injections of increasing amounts

of the allergenic extracts of the appropriate pollen or pollen-mixtures over prolonged periods lasting usually 3 to 5 years. Most of the pollen extracts used therapeutically are crude mixtures of a multiplicity of chemical constituents, some of these components bearing no relation whatsoever to the few allergenic components which are actually responsible for a given patient's hypersensitivity. Because some of the proteins present in these extracts may not be allergens, standardization of allergenic extracts based on total protein content is an unreliable guide for determining the potency of an extract. Moreover, large (up to 100x) variations in allergen content occur in the preparations used for immunotherapy because of the different methods used for (i) pollen collection and storage, which lead to variations in raw materials from lot to lot and from year to year, and (ii) the extraction procedures. Moreover, although different patients may be allergic to different constituents of a given pollen, all patients receive injections of the "same" complex mixture containing all the constituents of different pollens, i.e., they receive even components to which they may not be allergic. It is, therefore, not surprising that treatment with an ill-defined pollen extract may lead to the induction of additional IgE antibodies, i.e., to sensitization of the patients to new components (refs. 2 to 6).

While up to 80% of patients gain clinical improvement from this immunotherapy (refs. 7, 8), the risk of side effects, the lengthy course of therapy, the inconvenience to the patient of the mode and frequency of administration, and the mounting costs of this treatment limit the utility of the current immunotherapy. Although, local and systemic reactions may occur as a result of this therapy, they may be managed by a physician specialized in allergy. However, occasionally this mode of treatment is associated with the risk of

severe asthmatic or anaphylactic reactions, which can result in death (refs. 9, 10).

To eliminate some of the above disadvantages of the allergenic preparations currently used for immunotherapy, one of the major objectives in allergy research has been the isolation and characterization of the individual allergens of the complex repertoire of allergens of a given pollen by physicochemical and immunochemical methods. Several laboratories have isolated some of the allergens from the crude aqueous extracts of grass pollens by the use of classical physiochemical methods and reverse immunosorbents consisting of immobilized murine monoclonal antibodies to the pollen constituents (refs. 11, 12). Although such immunochemical methods appear to be promising for the characterization of individual allergens, the main drawback of these extremely labour intensive purification methods is the minute yield of allergens. Moreover, these methods do not ensure absolute purity of the allergenic constituents and, therefore, the determination of their amino acid sequences is difficult, if not impossible. As a corollary, the development of new therapeutic derivatives of grass pollen allergens and of reliable diagnostic procedures for pollen allergies are severely restricted by the use of allergens isolated by the existing procedures. However, recent innovations in recombinant DNA (rDNA) technology have paved the way for the synthesis of allergenic proteins, and of their epitopic fragments responsible for their activation of the appropriate B and T cells leading interactively to IgE formation, on an industrial scale and in a consistently pure state.

Upon initial exposure to allergenic constituents, they are internalized by antigen-presenting cells (APC), which include nonantigen-specific phagocytic cells or specific B cells, and are "processed" by these cells.

The net effect of this processing is the breakdown of the antigens into peptidic determinants which, in turn, are re-expressed in association with class I or class II molecules of the major histocompatibility complex (MHC) on the surface of the APC. Subsequently, the binary peptide-MHC complexes interact with the corresponding specific receptors (TCR) of, respectively, (i) Th cells or (ii) CTLs or Ts cells, and the resulting triads determine the up- or down-regulation of the appropriate B cells (ref. 13).

On the basis of their lymphokine secretion patterns, the Th cell subpopulation may be further subdivided into three subsets, i.e., Th0, Th1 and Th2 cells (ref. 14). In mice and man, the Th2 cells have been shown to produce IL-4, IL-5 and IL-6, and IL-4 has been shown to activate B cells leading to the production of IgE antibodies. In contrast, the Th1 cells produce IFNγ, which blocks the production of IL-4. The mechanism(s) involved in the suppression of antibody responses by Ts cells is still not fully understood. It has been suggested that the suppression of antibody production is due to inactivation of specific Th cells as a result of non-professional APC, i.e. a T-cell (ref. 15).

The primary reaction of the IgE antibodies secreted from the Bϵ cells is their binding to specific IgE receptors on the surface of mast cells, basophils and eosinophils. On re-exposure of the patient to the specific multivalent allergen, the cell-fixed IgE antibodies react with and are crosslinked by the allergenic molecules. This process leads to the release from these cells of chemical mediators of anaphylaxis. In turn, these mediators act rapidly on the smooth muscles of different target organs, resulting in the inflammatory manifestations characteristic of immediate type hypersensitivity.

The development of therapeutic strategies (ref. 16) that may influence the formation of IgE antibodies requires detailed knowledge of (i) the structures of individual allergens and, in particular, of their B cell epitopes (which are recognized by IgE/IgG antibodies), (ii) structures recognized by MHC molecule (i.e., Ia epitope), and (iii) the structures recognized by T cell receptors of Th or Ts cells, known as T cell epitopes. Therefore, determination of primary sequences of individual pollen allergens of a complex repertoire of allergens of a given pollen by immunochemical and physiochemical methods is of central importance, and has been a major objective in allergy research for a long time. However, using these classical methods, the progress in isolation and characterization of allergens has been slow. For example, for ragweed pollen, which is one of the main allergenic pollens in North America, in spite of intensive studies of over 50 years only six allergens (i.e., *Amb a* I, *Amb a* II, *Amb a* III, *Amb a* V, *Amb a* VI and *Amb t* V) have been purified and characterized (refs. 17 to 19). Similarly among grass pollens, one may cite pollens of Ryegrass, Timothy grass and Kentucky Bluegrass, and many others, which have been used for the isolation and characterization of their protein allergens (refs. 20 to 25).

Similar to other protein antigens, the epitopes of allergenic proteins are either continuous or discontinuous. Generally, continuous antigenic epitopes can be localized to segments composed of amino acid residues in a linear sequence, whereas discontinuous (conformational) epitopes comprise residues which appear adjacent to one another on the protein surface, but are widely separated in their primary sequence. The latter epitopes depend on the native conformation of the protein. Conventional methods for identification of B cell epitopes consist of probing a polyspecific antiserum

or a set of monoclonal antibodies produced against the intact antigen with cleavage fragments of antigen or synthetic peptides, which may yield information on continuous epitopes (refs. 26 to 27). Thus, on the basis of amino acid sequence data for Amb a III, Atassi and his associates synthesized ten overlapping pentadecapeptides which represented the entire Amb a III molecule (ref. 28). These peptides served to localize four antigenic sites in Amb a III that were recognized by IgG antibodies in human, rabbit and murine antisera. It is noted that the same regions were also recognized by human IgE antibodies (ref. 29).

Furthermore, by coupling partially-purified ragweed pollen allergens to monomethoxypolyethylene glycol (mPEG), it was shown that the resulting conjugates were (i) not only devoid of allergenicity and immunogenicity of the original ragweed pollen constituents in patients and mice, respectively, (ii) but were also capable of inducing a long-lasting suppression of IgE antibodies in mice (ref. 30). It was also reported that conjugates of poly-(N-vinyl pyrrolidone) with Timothy grass pollen allergens suppressed the established IgE responses to these allergens in mice (ref. 30).

Because of the limitation of the above classical purification methods which yield only minute amounts of pure allergens, recently some investigators have used the rDNA methods for the study of allergens. Thus, allergens present in dust mite (refs. 31 to 33), hornet venom (ref. 34), birch pollen (ref. 35) and grass pollens (ref. 36) have been cloned and the respective allergens produced by the application of rDNA techniques.

On the basis of sequence homology and cross-reactivities, the grass allergens cloned to-date may be classified to two main groups, one group includes allergens of 11 and 35 kDa in size (ref. 13) and the other group includes allergens of 28 to 34 kDa in size.

Published Canadian patent application No. 2,066,801, in which we are named as coinventors (corresponding to copending United States patent applications Serial No. _____ filed _____, the disclosure of which is incorporated herein by reference) discloses the cloning of the cDNAs of three isoallergens of Kentucky Blue Grass Poa pratensis (KBG) pollen and identified immunologically important regions within these latter group of allergens. These allergens are present in some other grasses and are thus useful for diagnosis of and desensitizing for grass-specific allergies.

As explained above, pollens of a wide variety of grasses and other plants produce aeroallergens. Therefore, it would be desirable to provide a cross-reactive allergen that is present in many grasses and other plant pollens. It would also be useful to provide the cDNA clone of such a cross-reactive allergen.

SUMMARY OF INVENTION

The present invention is directed toward improvements in allergen-specific immunotherapy by providing the means for overcoming the lack of pure allergens or peptides corresponding to portions thereof, as referred to above, by the determination of DNA sequences coding for a novel group of proteins of plant pollens. This group of allergens, though constituting major allergenic components of plant pollens, remained uncloned prior to this invention.

Accordingly, the present invention provides, in one aspect, a purified and isolated nucleic acid molecule comprising at least a portion coding for an allergenic protein which is present in pollens from both monocotyledonous and dicotyledonous plants.

The monocotyledonous plants are generally those selected from the family Gramineae, particularly grasses including Bermuda grass, Kentucky Blue grass, Red Top grass, Reed Canary grass, Rye grass, Timothy grass, Brome

grass, Orchard grass and cultivated corn. The dicotyledonous plants include trees, for example, birch, and weeds, for example, ragweed, particularly short ragweed, and parietaria.

The portion of the nucleic acid molecule coding for the allergenic protein provided herein generally is selected from:

(a) the DNA molecule including the DNA sequence set out in Figure 1 or its complementary strand;

(b) a DNA molecule encoding a protein including the amino acid sequence set forth in Figure 2; and

(c) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (a) or (b), and is conserved among the monocotyledonous and dicotyledonous plants. Preferably, the DNA sequences defined in (c) have at least about 75% sequence identity with the sequence defined in (a) or (b).

In another aspect of the invention, the present invention provides a recombinant plasmid adopted for transformation of a host, comprising a plasmid vector into which has been inserted a DNA segment comprising at least a 15 bp fragment of a DNA molecule as provided herein. The present invention also includes an expression vector adapted for transformation of a host, comprising at least a DNA segment comprising at least a 15 bp fragment of a DNA molecule as provided herein and expression means operatively coupled to the DNA segment for expression thereof in a host. In particular, the DNA segment encodes an allergenic protein including the amino acid sequence of Figure 2.

The DNA segment contained in the expression vector may further comprise a nucleic acid sequence encoding a carrier protein, for example, glutathione-S-transferase (GST), β-galactosidase or protein A, for expression of a carrier-allergen fusion. One particular expression vector provided herein is plasmid pNH1, deposited with

the American Type Culture Collection, Rockville, Maryland, U.S.A., on January 10, 1994 and having ATCC accession number 75,634.

An additional aspect of the invention provides a recombinant protein produced by expression in the host of the DNA fragment contained in the expression vector provided herein or a functional analog of the protein. In this application, a first protein is a "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution addition or deletion mutant thereof. The present invention also includes synthetic allergenic or antigenic peptides corresponding in amino acid sequence to portions of the recombinant protein or allergen.

The present invention provides, in a further aspect, a composition for protecting allergic individuals from developing an allergic reaction, comprising at least one active component selected from at least one nucleic acid molecule and at least one recombinant protein, provided in accordance with aspects of the invention, and a pharmaceutically-acceptable carrier therefor, particularly formulated for in vivo administration.

In such immunogenic compositions, the composition may comprise at least one recombinant protein conjugated to a non-immunogenic substrate, which may be a polymeric material, for example, a carboxymethyl cellulose, a monomethoxypolyethylene glycol and a polyvinyl alcohol. The non-immunogenic substrate may comprise beads for targeted uptake of the at least one recombinant protein by antigen-presenting cells.

The composition may be formulated as a microparticle, capsule or liposome preparation and may be provided a combination with a targeting molecule for

delivery to specific cells of the immune system or to mucosal surfaces.

The composition provided in this aspect of the invention may be combined with at least one additional desensitizing agent, which may be selected from Poa p IX allergen, Lol p I allergen, Bet v I allergen, Amb a I allergen and Amb a II allergen. In addition, the composition may comprise at least one compound having anti-histamine activity and/or at least one compound having anti-inflammatory activity and/or at least one compound which is immunosuppressive. The composition also may comprise an adjuvant.

In an additional aspect, the present invention provides a method for desensitizing an allergic individual, particularly a human, by administering an effective amount of the composition provided herein, particularly one containing the at least one additional desensitizing agent.

The present invention provides, in a further aspect, a method of depleting allergen-specific antibodies from an individual, particularly a human, by contacting the antibodies with the composition provided herein to form a complex, and removing the complex from the individual.

In another aspect, the present invention provides a method of anergizing allergen-specific antibody-producing cells, particular in a human, by contacting the cells with the composition provided herein.

The present invention also provides, in an additional aspect, an antiserum specific for a recombinant protein as provided herein.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the nucleotide sequence (SEQ ID NO: 1) and the deduced amino acid sequence (SEQ ID NO: 2) of a portion of a cDNA clone (KBG 51) obtained by screening a Kentucky Bluegrass pollen - λgt11 library;

Figure 2 contains Northern blot analysis of KBG pollen mRNA using KBG 30 as lane 1, KBG 51 as lane 2 and KBG 60 as lane 3 insert DNAs as probes;

Figure 3 illustrates detection of homology in mRNA in a number of pollen by RT-PCR followed by Southern analysis using KBG 51 cDNA as the probe;

Figure 4 illustrates vector plasmid pNH1 and the cloning site for KBG 51;

Figure 5 illustrates high-level expression of KBG 51 in the pGEX-2T-1 system; and

Figure 6 contains Western blots of a variety of pollen extracts utilizing antiserum to GST-KBG 51 fusion protein.

GENERAL DESCRIPTION OF INVENTION

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of diagnosis and therapy of allergic diseases, such as allergic rhinitis, asthma, food allergies and atopic eczema. In a diagnostic embodiment, the demonstrated cross-reactivity of the allergen is particularly useful. Thus, as described above, the usual method of determining to which aeroallergens an individual is allergic involves an intra-dermal exposure to many crude extracts of allergen extracts (for example, mixtures of grasses, weeds and trees). This procedure involves the use and administration of many allergens. The cross-reactive allergen of the present invention (specifically CRAL51) now allows for the demonstration of IgE antibodies against a whole range of allergens. An allergic individual who has CRAL51 specific IgE/antibodies can now be immediately excluded from being allergic to this whole range of allergens. A further non-limiting discussion of such uses is further presented below.

Preparation and Use of Composition for Protecting Allergic Individuals for Developing an Allergic Reaction

Compositions, suitable to be used for protecting allergic individuals from developing an allergic reaction, may be prepared from CRAL51 allergen, analogs, fragments and/or peptides as disclosed herein. Compositions may be prepared as injectables, as liquid solutions or emulsions. The CRAL51 allergen fragment analogs or peptides may be mixed with pharmaceutically-acceptable excipients which are compatible with the allergen proteins, fragment analogs or peptides. Excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The composition may further contain minor amounts of auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Methods of achieving an adjuvant effect for the compositions includes the use of agents, such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1 percent solution in phosphate buffered saline. Compositions may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10 to about 95% of the allergen fragment analogs and/or peptides.

The compositions are administered in a manner compatible with the dosage formulation, and in such

amount as is therapeutically effective to protect allergic individuals from developing an allergic reaction. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies. Precise amounts of allergen required to be administered depends on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of nanograms to micrograms of the allergen, analog fragment and/or peptides. Suitable regimens for initial administration and booster does are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the composition may also depend on the route of administration and will vary according to the size of the host.

As described above, the cross-reactive allergen may be conjugated to a non-immunogenic substrate including polymeric materials, such as carboxymethyl celluloses, monomethoxypolyethylene glycols (mPEG) and polyvinyl alcohols, to render it non-immunogenic and non-allergenic for protecting allergic individuals from developing an allergic reaction. mPEG conjugates to a variety of allergens have been tested in human clinical trials (ref. 45). The availability of a cross-reactive allergen in a purified form in large amounts now makes it possible to synthesize well defined conjugates for these molecules.

The nucleic acid molecules encoding the allergen of the present invention or portions thereof may also be used directly for immunization by adminstration of the DNA directly, for example, by injection for genetic immunization or by constructing a live vector, such as Salmonella, BCG, adenovirus, poxvirus, vaccinia or poliovirus. A discussion of some live vectors that have been used to carry heterologous antigens to the immune

system are discussed in, for example, O'Hagan (1992 - ref. 46). Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer et al., 1993 (ref. 47).

The use of peptides corresponding to portions of the cross-reactive allergen in vivo may first require their chemical modification, since the peptides themselves may not have a sufficiently long serum and/or tissue half-life. Such chemically modified peptides are referred to herein as "peptide analogs". The term "peptide analog" extends to any functional chemical equivalent of a peptide characterized by its increased stability and/or efficacy in vivo or in vitro in respect of the practice of the invention. The term "peptide analog" is also used herein to extend to any amino acid derivative of the peptides as described herein. Peptide analogs contemplated herein are produced by procedures that include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of cross-linkers and other methods which impose conformational constraint on the peptides or their analogs.

Examples of side chain modifications contemplated by the present invention include modification of amino groups, such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acetylation with acetic anhydride; carbamylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulfonic acid (TNBS); alkylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxa-5'-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation

products with reagents, such as 2, 3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via o-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulfhydryl groups may be modified by methods, such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of mixed disulphides with other thiol compounds; reaction with maleimide; maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulfonic acid, phenylmercury chloride, 2-chloromercuric-4-nitrophenol and other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tryosine residues may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid-, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

The purified and isolated DNA molecules comprising at least a portion coding for an allergenic protein which is present in pollens from both monocotyledonous and

dicotyledonous plants typified by the embodiments described herein are advantageous as:

- nucleic acid probes for the specific identification of other allergens that contain a DNA sequence comprising at least a portion coding for the cross-reactive allergen CRAL51 or analogs thereof;

- the products encoded by the DNA molecules are useful as diagnostic reagents to identify CRAL51 specific IgE antibodies in an individual to demonstrate or exclude allergy to a whole range of allergens;

- peptides corresponding to portions of the allergen as typified by the embodiments described herein are advantageous as diagnostic reagents, antigens for the production of allergen-specific antisera, for example, for the demonstration of an allergic reaction to a whole range of allergens.

Immunoassays

The cross-reactive allergen, analog, fragment and/or peptide of the present invention are useful as immunogens, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of allergen specific IgE antibodies. In ELISA assays, the allergen, analog, fragment and/or peptide corresponding to portions of the allergen are immobilized onto a selected surface, for example, a surface exhibiting a protein affinity, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed allergen, analog, fragment and/or peptide, a nonspecific protein, such as bovine serum albumin (BSA) or casein that is known to be antigenically neutral with regard to the test sample, may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the

surface. Normally, the peptides are at least 12 residues in length and preferably 14 to 30 residues. It is understood however, that a mixture of peptides may be used either as an immunogen in a composition or as a diagnostic agent.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to about 4 hours, at temperatures, such as of the order of about 25° to about 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound allergen, analog, fragment and/or peptide, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody would be an antibody having specificity for human IgE or IgG antibodies. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then achieved by measuring the degree of color generation using, for example, a visible spectra spectrophotometer.

Use of Sequences as Hybridization Probes

The nucleotide sequences of the present invention, comprising the sequence of the cross-reactive allergenic protein, now allow for the identification and cloning of the allergenic protein genes from other sources.

The nucleotide sequences comprising the sequence of the allergenic protein of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other allergenic protein genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other allergenic protein genes. For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results.

A wide variety of appropriate indicator means are known in the art for determining hybridization, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of providing a detectable signal. In some embodiments, an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific

hybridization with samples containing allergenic protein gene sequences.

The nucleic acid sequences of allergenic protein genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid phase procedures, the test DNA (or RNA) from samples, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the allergenic protein genes or fragments thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. The selected probe should be at least 18 bp and is preferably in the range of 30 bp to 90 bp long.

Expression of the Allergenic Protein Genes

Plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the allergenic protein genes in expression systems. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters

which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978 (ref. 48): Itakura et al., 1977 (ref. 49); Goeddel et al., 1979 (ref. 50); Goeddel et al., 1980 (ref. 51)) and other microbial promoters, such as the T7 promoter system. Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with plasmid vectors. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the allergen genes, fragments, analogs or variants thereof include *E. coli*, *Bacillus* species, fungi, yeast, higher eukaryotic cells, such as CHO cells, or the baculovirus expression system may be used.

In accordance with this invention, it is preferred to make the allergenic protein, fragment or analog by recombinant methods. Particularly desirable hosts for expression include Gram positive bacteria which do not have LPS and are therefore endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of allergenic protein, fragments or analogs thereof.

The allergenic protein may also be produced as a fusion protein with, for example, glutathione-S-transferase, β-galactosidase and protein A.

Biological Deposits

A plasmid pNH1 (pUM 94.1) that contains at least a portion coding for an allergenic protein that is described and referred to herein has been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland USA pursuant to the Budapest Treaty on January 10, 1994 and has been accorded accession number 75,634. A further plasmid pUM 94.2 (KBG 15) also containing at least a portion coding for an allergenic protein has been deposited with ATCC on _____ and has been accorded accession number _____. Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed herein is not to be limited in scope by the plasmid deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations. Immunological and recombinant DNA methods may not be explicitly described in this disclosure but are well within the scope of those skilled in the art.

EXAMPLES

Example 1

This Example describes the cloning and sequencing of a cDNA encoding a portion of the cross-reactive allergen (CRAL 51).

Certain aspects of the cloning of allergen-encoding cDNAs is described in the aforementioned Canadian patent application No. 2,066,801 (and corresponding USA 08/_____).

Total pollen RNA was extracted from 35 g of mature KBG pollen (Hollister-Stier, Miles Laboratories, Toronto) using the guanidine isothyocyanate-cesium chloride method (ref. 37). Scanning electron microscopy confirmed that the pollen was that of Poa pratensis and was free of any contaminants. Poly(a)+RNA was prepared by affinity chromatography on poly-U Sepharose (Pharmacia, Toronto).

Plaques (250,000) from a λgt11 library (ref. 38) were screened immunologically for the expression of pollen allergens using pooled sera of eight patients allergic to KBG pollen. Patients were selected on the basis of case history, positive skin prick and radioallerosorbant tests but had not undergone any hyposensitization treatments. Plaques were lifted onto nitrocellulose filters (Bio-Rad, Toronto) and washed twice for 5 and 30 minute intervals in 30 mls of buffer (50 mM sodium phosphate, pH 7.5, 0.5% v/v Tween 20, 0.5% bovine serum albumin (BSA), 0.05% sodium azide w/v) per filter. The filters were then allowed to hybridize overnight at 4°C under slight agitation in buffer containing 0.01% pooled sera. The filters were then washed at 4°C, for three 15 minute intervals in 25 ml of buffer per filter, and incubated overnight at 15°C with goat-anti-human IgE conjugated with alkaline phosphatase. The filters were re-washed as previously described and developed using 5-bromo-4-chloro-3-indolylphosphate p-toluidine and nitroblue tetrazolium chloride. Positive clones were picked and re-screened, one of these clones, (KBG51) was further characterized.

DNA was prepared from plaque purified phage using the liquid lysate method (ref. 39). Inserts recovered from *Eco* RI digestion were ligated into the multiple

cloning site (MCS) of pBluescript and used to transform *E. coli* DH5α.

The sequence of the cDNA clone KBG51 (SEQ ID NO: 1) and the deduced amino acid sequence (SEQ ID NO: 2) are shown in Figure 1. cDNA KBG51 is a partial cDNA clone with an open reading frame. The deduced polypeptide comprises 145 amino acids with an estimated Mr of 16.636 kDa and pI of 12.02. Comparison of the nucleotide and deduced amino acid sequences with the GenBank DNA and PIR protein sequence Data Banks, respectively indicated that the sequences were unique and not homologous to any other known allergen or non-allergen DNA or proteins in these Data Banks. Thus, the cloned cDNA encodes a novel allergen which has not been described previously.

Example 2

This Example describes the demonstration of mRNA species in KBG pollen corresponding to the KBG51 cDNA.

Total pollen and leaf RNA was electrophoresed in 1.5% agarose gels under dewatering conditions (ref. 40) and blotted onto nylon membrane.

Northern blot hybridization using radiolabelled KBG51 cDNA insert revealed that the corresponding transcripts are present in KBG pollen but not in leaf tissue (Figure 2). Northern hybridization using cDNA probes corresponding to allergens of two other cDNAs belonging to Poa p IX used in this experiment as controls indicated that transcripts corresponding to these cDNAs were maximally 1.5 kb in length. The transcripts of this size has also not been reported previously for any grass allergen cDNAs. On the basis of the size of this transcript it is concluded that these transcripts encode a group of allergens, cDNAs of which have not yet been described.

The results of this analysis are shown in Figure 2 and demonstrate that mRNA transcripts of about 2.0 kb and about 1.4 kb (lane 2 of Figure 3) were detectable in

pollen mRNA but not in leaf mRNA from KBG pollen. Furthermore, these mRNA species differed from those described in Canadian patent application No. 2,066,801 as shown in lanes 1 (KBG 31) and 3 (KBG 60).

Example 3

This Example demonstrates the presence of transcripts corresponding to KBG51 in a variety of other pollens.

Total RNA prepared from the pollens indicated in Figure 3 as described in Example 1 was subjected to reverse transcription reaction. Thus, the first strand cDNA was synthesized in a 50 μl final volume at 42°C for 1 h using a 5 μg of total RNA, reverse transcriptase and oligo dT primer. The double strand cDNA was synthesized using DNA polymerase and dNTP according to the manufacturers instructions (BRL, Mississauga, ON). The products obtained were heat denatured and an aliquot of 5 μl was utilized in amplification reaction with specific primers (DNA Synthesis Laboratory, Univ. of Manitoba) for the KBG51 cDNA. The primers are shown on the DNA sequence of Figure 1 and had the sequences KBG, forward 5' - TCT TGG CTT GAC CGA AGC (SEQ ID NO: 3) and KBG, reverse 5' - GAT ACA GCC CAT CAC CGC (SEQ ID NO: 4).

The PCR amplification (ref. 41) was carried out in a buffer containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 200 μM dNTP, 2.5 Taq polymerase (Cetus Perkin Elmer) and 50 pmol of each primer. The reaction consisted of 30 cycles of amplification followed by a 15 min. final extension at 72°C. Each cycle included denaturation at 95°C for 30 sec, annealing at 65°C for 60 sec. and extension at 72°C for 2 min. The amplified products were then precipitated with ethanol, run on an agarose gel, transferred to nylon membranes and hybridized under stringent conditions with random-primed KBG51 cDNA insert. The results of this analysis are shown in Figure 3 and indicate that mRNA

from all pollens tested contained a transcript corresponding to KBG51. This analysis demonstrated that the allergen encoded by KBG51 is present in a wide variety of plant pollens and is therefore a cross-reactive allergen. Analysis of nucleotide sequences of the PCR fragments indicated that the sequences of the amplified cDNAs were identical to that of clone 51.

Example 4

This Example describes the production of a GST-KBG51 fusion protein.

For identification of the polypeptides encoded by the KBG51 cDNA clone, the insert was cloned in the pGEX 2T-1 expression vector (ref. 42) to produce plasmid pNH1 (Figure 4) which permitted high-level expression of the GST-KBG51 fusion protein (Figure 6). The fusion protein was expressed as a soluble fusion protein of approximately 43 kDa in conjunction with the glutathione-S-transferase (GST), which upon cleavage yielded the GST of about 26 kDa. The protein expression was estimated to be about 20% of the total cell extract or about 2 mg/g wet wt of cells. Total crude proteins were recovered form the host cells and the fusion protein was purified by electrophoresis on 10% SDS-PAGE followed by electroelution from the polyacrylamide gel to produce the GST-KBG51 fusion protein as shown in lane 3 of Figure 5.

Example 5

This Example describes the generation of GST-KBG51 fusion protein specific antiserum and its cross reactivity to proteins in other plant pollens.

The GST-KBG51 fusion protein with dextran sulfate as adjuvant, was used as immunogen to immunize 6 to 8 week old female BDF1 mice (Jackson Laboratories, Bar Harbour Michigan). The mice were immunized subcutaneously and after 23 days the mice were boosted with the same antigen preparation. Blood was collected at day 30 after primary immunization and serum was prepared.

Proteins from various pollens (as shown in Figure 6) were isolated as previously described (ref. 17) and electrophoresed on a 15% polyacrylamide gel. Proteins were then stained with Coomassie Blue or electroblotted onto a nitrocellulose membrane. Membranes were blocked with 1% gelatin in TBS (10 mM Tris-HCl pH7.5, 150 mM NaCl) and probed with the antiserum raised against GST-KBG51. Immunoreactive bands were detected using an alkaline phosphatase conjugated goat anti-mouse second antibody.

A Western blot analysis (ref. 43) employing the polyclonal murine antiserum to the GST-KBG51 fusion protein led to the identification of two components in the KBG extract, a major component having an approximate Mr of 59 kDa and a minor component having an approximate Mr of 30 kDa (Figure 6 lane 3 and Table 1 (the Tables appear at the end of the disclosure)). A Western blot analysis of a number of pollen extracts showed that a number of polypeptides in these extracts reacted with the antiserum indicating the presence of cross-reactive allergens in these pollens. Table 1 summarizes the polypeptides recognized by the antiserum in protein extracts prepared from a number of different grasses, weeds and tree pollens. This antibody recognized two high molecular weight polypeptides in the ryegrass pollen extract which has not been reported previously. From these studies it is concluded that the allergen encoded by the KBG51 cDNA comprises a very highly cross-reactive group of allergens in plant pollens.

Example 6

This Example describes the percentage of the allergic population that have KBG51 specific IgE antibodies.

The sera of approximately 1000 individuals who were allergic to a broad spectrum of allergens, including

pollen, were assessed for their ability to bind GST-KBG51 fusion protein in an ELISA assay (ref. 44).

GST-KBG51 protein (10 μg/ml) was coated to wells of microtiter plates (Nunc, Immunoplate, Denmark) overnight. The wells were further blocked with phosphate buffer containing 1% gelatin for 8 hrs. Subsequent to washing 5 times with phosphate buffer containing 0.05% Tween 20, the wells were incubated with human serum diluted with the washing buffer containing 0.1% gelatin overnight. After washing, alkaline phosphatase-conjugated secondary antibodies (goat anti-human IgE (TAGO), diluted 300-fold) were added and the wells were incubated overnight. Following washing with phosphate buffer containing Tween 20, the color reaction was developed by adding the substrate para-nitro phenyl phosphate (1 mg/ml) in 0.1 M Tris-HCl buffer containing NaCl and $MgCl_2$ and the OD was measured at 405 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, VA).

The results of the ELISA assays are shown in Table 2 and indicate that individuals from a variety of geographical areas possessed IgE antibodies that recognized the GST-KBG51 fusion protein. This indicates that the individuals have been sensitized to CRAL51, portions or analogs thereof and suggests that CRAL51 allergen is a broadly cross reactive allergen present in many aeroallergens to which individuals are routinely exposed.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides certain novel nucleic acid molecules which code for allergenic proteins present in a wide spectrum of plant pollens. Modifications are possible within the scope of this invention.

Table 1

Summary of components recognized by antiserum to GST-KBG51 fusion protein in different pollen extracts

Pollen	KBG	Canary	Colonial	Cultivated corn	Johnson	Orchard	Perennial rye	Quack	Smooth-brome
Approximate Molecular Weight (kDa)	59 30	115 65 46.5	80.5 54 34	64 40.5 30	47 34	80.5 69 65 58.5 29.5	81 57 42 31	80 57 46 33.5	81 57.5 46 32

Pollen	Rye	Bermuda	Birch	Parietaria	Red Top	Tall	Timothy	Barley	Reed Canary	Smooth
Approximate Molecular Weight (kDa)	94 81	105 88 53 30	89 67 35	100 43	81 67 56 30	81 65 45 35	63 35	74 54 35	56	78 52

Table 2

Human IgE binding of GST-KBG51 fusion protein using a panel of international sera by ELISA

Laboratory Number (Country)	Number of sera examined	Number of IgE positive sera with specificity for KBG extract	Number of IgE positive sera with specificity for GST-KBG51 fusion protein
5 (U.S.A.)	98	16	16
466-03 (U.S.A.)	20	7	1
1351-10 (U.S.A.)	23	7	5
489-18 (U.S.A.)	100	27	14
397-27	9	4	2
315-06 (Columbia)	89	17	26
13 (Japan)	100	26	17
20 (Japan)	33	10	4
33 (Japan)	33	16	11
36 (Japan)	71	25	8
1487-35 (Taiwan)	17	0	2
19 (Denmark)	25	14	0
4 (Sweden)	94	43	21
233-26 (Italy)	67	29	11
233-28 (Italy)	36	22	16
1488-34 (Bulgaria)	65	39	18
11	37	11	10
25 (Israel)	30	8	6

REFERENCES

1. Firedhoff LR. In: Genetic and Environmental factors in Clinical Allergy, Marsh DG and Blumenthal MN (eds.) Univ. of Minnesota Press (1989).
2. Loca AF and Cooke RA. J. Immunol. 8: 162 (1923).
3. Richter, M. *et al.* J. Allergy 29: 298 (1958).
4. Marsh, DG. *et al.* Immunology 22: 1013 (1972).
5. Lichtenstein, LM *et al.* In: 11th Int. Congr. of Allergology and Clin. Immunol. Kerr Jw and Ganderton MA (eds.) pp. 285 (1983).
6. Hamilton RG. Curr. Opinions in Immunol. 2: 558 (1990).
7. Bousquet J. *et al.* J. Allergy Clin. Immunol. 84: 546 (1989).
8. Creticos PS *et al.* J. Allergy Clin. Immunol. 84: 197 (1989).
9. Kay AB. Clin. Exp. Allergy 19: 591 (1989).
10. CMS Update: Desensitizing vaccines Brit. Med. J. 293: 948 (1986).
11. Ekramoddoullah AKM. *et al.* Int. Arch. Allergy Clin. Immunol. 80:100 (1986).
12. Kahn CR and Marsh DG. Fed. Proc. 41: 826 (1982).
13. Mohapatra, S.S. In : Clinical Reviews in Allergy, Special topic on "New therapeutic issues on bronchial asthma", T. Nakagawa(ed), Humana press (in press)(1994).
14. Romagnani, S. Immunology Today 11:316 (1990)
15. Mohapatra, S et al. J.Immunology 151:688-698 (1993)
16. Mohapatra, S.S. Pharmacia Allergy Research Foundation Awards Book, PP.4-15, (1992)
17. King TP. In: Proc. 8th Int. Congr. Allergology Clin. Immunol. Mounro-Ashman (ed.), Elsevier pp. 394 (1974)
18. Roebber M. *et al.* J. Immunol. 131: 706 (1983).
19. Smitt JJ. *et al.* Mol. Immunol. 25: 355 (1988).
20. Ford SA, Baldo BA. Int. Arch. Allergy Appl. Immunol. 81: 193 (1986).
21. Johnson P, Marsh DG. Eur. Polymer J. 1: 63 (1965).44.
22. Johnson P, Marsh DG. Immunochemistry 3: 91 (1966).

23. Johnson P, Marsh DG. Immunochemistry 3: 101 (1966).
24. Ansari AA. *et al.* J. Biol. Chem. 264: 11181 (1989).
25. Ansari AA. *et al.* Biochemistry 28: 8665 (1989).
26. Jaggi KS. *et al.* J. Allergy Clin. Immunol. 83: 845 (1989).
27. Esch RE and Klapper DG. Mol. Immunol. 26: 557 (1989).
28. Atassi H and Atassi MZ. Eur. J. Immunol. 16: 227 (1986).
29. Kurisake J. *et al.* Eur. J. Immunol. 16: 236 (1986).
30. Sehon AH. Prog. Allergy 32: 161 (1982).
31. Chua KY. *et al.* J. Exp. Med 167: 175 (1988).
32. Chua KY. *et al.* Int. Arch. Allergy Clin. Immunol. 85: 127 (1988).
33. Tovey ER, Johnson MC, Roche AL, Cobon GS, Baldo BA. J. Exp. Med. 170: 1457 (1989).
34. Fang KSY. *et al.* Proc. Natl. Acad. Sci. USA 85: 895 (1988).
35. Breitenader H. *et al.* EMBO J. 8: 1935 (1989).
36. Mohapatra SS. *et al.* Int. Arch. Allergy Appl. Immunol. 91: 362 (1990).
37. Chirgwin, J.M. et al. Biochemistry 18:5294 (1979).
38. Kaslow DC. Nucleic Acids Res. 14: 6767 (1986).
39. Tabor S and Richardson CC. Proc. Natl. Acad. Sci. U.S.A. 84: 4767 (1987).
40. Alwine JC. *et al.* Proc. Natl. Acad. Sci. U.S.A. 74: 5350 (1977).
41. Mullins et al, U.S. Patents Nos. 4,683,202 and 4,800,159.
42. Olsen, E. and Mohapatra S.S. Int. Arch. Allergy Immunol. 98:343-348 (1992)
43. Zhang I. et al. Int. Arch. Allergy Appl. Immunol. 96:28-34 (1991)
44. Olsen E. and Mohapatra S.S., Ann. Allergy, (1994) (In press).
45. Dreborg S. and Akerbomb E., Lit. Review Ther. Drug Carrier Sept., CRC Press, vol. 6, p315-365 (1990).
46. O'Hagan (1992) Clin. Pharmokinet. 22:1 (1992).
47. Ulmer et al., (1993) Curr. Opinion Invest. Drugs. 2 (9): 983-989.
48. Chang et al., (1978) Nature 375:615.

49. Itakura et al., (1977) Science 198:1056.
50. Goeddel et al., (1979) Nature 281:544.
51. Goeddel et al., (1980) Nucl. Acids Res. 8:4057.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: CROSS-REACTIVE ALLERGEN

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 504 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:7..441

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GAATTC TTG CGC GAT GTT CTC GCC GCG CAG TGT CTT GAC CTT CTC GCC          48
       Leu Arg Asp Val Leu Ala Ala Gln Cys Leu Asp Leu Leu Ala
         1               5                  10

GTC GAT GAA CAC CGG CGC GGC CGG ATT CTC TCC CGA CCC CGG CAA GCT          96
Val Asp Glu His Arg Arg Gly Arg Ile Leu Ser Arg Pro Arg Gln Ala
 15                  20                  25                  30
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GAT GCC GAT ATT CGC CTG CTT CGA CTC GCC CGG ACC GTT GAC GAT ACA        144
Asp Ala Asp Ile Arg Leu Leu Arg Leu Ala Arg Thr Val Asp Asp Thr
                35                  40                  45

GCC CAT CAC CGC CAC GTG CAT CTT CTC GAC GCC AGG ATA CTG ATC GCG        192
Ala His His Arg His Val His Leu Leu Asp Ala Arg Ile Leu Ile Ala
            50                  55                  60

CCA CAC CGG CAT CTG CGT ACG CAG ATA GGT CTG GAT TTG CGA CGC CAG        240
Pro His Arg His Leu Arg Thr Gln Ile Gly Leu Asp Leu Arg Arg Gln
        65                  70                  75

TTC CTG GAA CAG TGT GCT GGT GGT GCG GCC ACA ACC CGG GCA CGC GAT        288
Phe Leu Glu Gln Cys Ala Gly Gly Ala Ala Thr Thr Arg Ala Arg Asp
    80                  85                  90

CAC CAT CGG CGT GAA CGA GCG CAG ACC CAT GGT CTG CAG GAT TTC CTG        336
His His Arg Arg Glu Arg Ala Gln Thr His Gly Leu Gln Asp Phe Leu
 95                 100                 105                 110

ACC GAC GAC CAC CTC GCC GGT GCG CGG GCC GCC CGG TTC CGG CGT CAG        384
Thr Asp Asp His Leu Ala Gly Ala Arg Ala Ala Arg Phe Arg Arg Gln
                115                 120                 125

CGA GAT GCG GAT CGT GTC GCC GAT GCC CTG CTG CAG CAA TAC GGA CAA        432
Arg Asp Ala Asp Arg Val Ala Asp Ala Leu Leu Gln Gln Tyr Gly Gln
            130                 135                 140

CGC AGC GGT TGACGCCACG ATGCCCTTGG AGCCCATGCC GGCTTCGGTC                481
Arg Ser Gly
        145

AAGCCAAGAT GCAGCGCGAA TTC                                             504
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 145 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Leu Arg Asp Val Leu Ala Ala Gln Cys Leu Asp Leu Leu Ala Val Asp
 1               5                  10                  15

Glu His Arg Arg Gly Arg Ile Leu Ser Arg Pro Arg Gln Ala Asp Ala
            20                  25                  30

Asp Ile Arg Leu Leu Arg Leu Ala Arg Thr Val Asp Asp Thr Ala His
        35                  40                  45

His Arg His Val His Leu Leu Asp Ala Arg Ile Leu Ile Ala Pro His
    50                  55                  60

Arg His Leu Arg Thr Gln Ile Gly Leu Asp Leu Arg Arg Gln Phe Leu
65                  70                  75                  80

Glu Gln Cys Ala Gly Gly Ala Ala Thr Thr Arg Ala Arg Asp His His
                85                  90                  95

Arg Arg Glu Arg Ala Gln Thr His Gly Leu Gln Asp Phe Leu Thr Asp
            100                 105                 110
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Asp His Leu Ala Gly Ala Arg Ala Ala Arg Phe Arg Arg Gln Arg Asp
115 120 125

Ala Asp Arg Val Ala Asp Ala Leu Leu Gln Gln Tyr Gly Gln Arg Ser
130 135 140

Gly
145

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCTTGGCTTG ACCGAAGC 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATACAGCCC ATCACCGC 18

CLAIMS

What we claim is:

1. A purified and isolated nucleic acid molecule, the molecule comprising at least a portion coding for an allergenic protein which is present in pollens from both monocotyledonous and dicotyledonous plants.

2. The nucleic acid molecule of claim 1 wherein said monocotyledonous plants are selected from the family Gramineae.

3. The nucleic acid molecule of claim 2 wherein said monocotyledonous plants are selected from Bermuda grass, Kentucky Blue grass, Red Top grass, Reed Canary grass, Rye grass Timothy grass, Brome grass, Orchard grass and cultivated corn.

4. The nucleic acid molecule of claim 1 wherein said dicotyledonous plants are selected from trees and weeds.

5. The nucleic acid molecule of claim 4 wherein said tree is a Birch tree and said weeds are selected from ragweed and parietaria.

6. The nucleic acid molecule of claim 5 wherein said ragweed is short ragweed.

7. The nucleic acid molecule of claim 1 wherein the portion coding for the allergenic protein is selected from:

(a) a DNA molecule including the DNA sequence set out in Figure 1 or its complementary strand;

(b) a DNA molecule encoding a protein including the amino acid sequence set out in Figure 2; and

(c) DNA sequences which hybridize under stringent conditions to the DNA molecules defined in (a) or (b), and is conserved among said monocotyledonous and dicotyledonous plants.

8. The nucleic acid molecule of claim 7 wherein said DNA sequences defined in (c) have at least about 75% sequence identity with sequences defined in (a) or (b).

9. A recombinant plasmid adapted for transformation of a host, comprising a plasmid vector into which has been inserted a DNA segment comprising at least a 15 bp fragment of a nucleic acid molecule of claim 7.

10. An expression vector adapted for transformation of a host, comprising at least a DNA segment comprising at least a 15 bp fragment of a nucleic acid molecule of claim 7 and expression means operatively coupled to the DNA segment for expression thereof in the host.

11. The expression vector of claim 10 wherein said DNA segment further comprises a nucleic acid sequence encoding a carrier protein for expressing a carrier-allergen fusion.

12. The expression vector of claim 11 wherein said carrier protein is selected from glutathione-S-transferase, β-galactosidase and protein A.

13. The expression vector of claim 10 which is plasmid pNH1 having ATCC accession number 75,634.

14. A recombinant protein produced by expression in said host of the DNA fragment contained in the expression vector of claim 10 or a functional analog of the protein.

15. A composition for protecting allergic individuals from developing an allergic reaction, comprising at least one active component selected from at least one nucleic acid molecule of claim 1 and at least one recombinant protein of claim 14, and a pharmaceutically-acceptable carrier therefor.

16. The composition of claim 15 formulated as a vaccine for *in vivo* administration.

17. The composition of claim 16 wherein said vaccine comprises said at least one recombinant protein conjugated to a non-immunogenic substrate.

18. The composition of claim 17 wherein said non-immunogenic substrate is selected from polymeric materials.

19. The composition of claim 18 wherein said polymeric material is selected from carboxymethyl celluloses, monomethoxypolyethylene glycols and polyvinyl alcohols.

20. The composition of claim 17 wherein said non-immunogenic substrate comprise beads for targeted uptake of said at least one recombinant protein by selected antigen-presenting cells.

21. The composition of claim 15 formulated as a microparticle, capsule or liposome preparation.

22. The composition of claim 15 in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces.

23. The composition of claim 16 comprising at least one additional desensitizing agent.

24. The composition of claim 23 wherein said at least one additional desensitizing agent is selected from Poa p IX allergen, Lol p I allergen, Bet v I allergen, Amb a I allergen and Amb a II antigen.

25. The composition of claim 16 further comprising at least one compound having anti-histamine activity, and/or at least one compound have anti-inflammatory activity and/or at least one compound which is immunosuppressive.

26. The composition of claim 15 further comprising an adjuvant.

27. The composition of claim 15 wherein said at least one nucleic acid molecule is contained in a vector.

28. The composition of claim 27 wherein the vector is selected from Salmonella, BCG, adenovirus, poxvirus, vaccinia or poliovirus.

29. A method for desensitizing an allergic individual, comprising administering to the individual an effective amount of the composition of claim 15.

30. The method of claim 29 wherein the individual is a human.

31. The method of claim 30 wherein said immunogenic composition is that of claim 24.

32. A method of depleting allergen-specific antibodies from an individual, comprising contacting said antibodies with said composition of claim 15 to form a complex, and removing the complex from the individual.

33. The method of claim 32, wherein said individual is a human.

34. A method of anergizing allergen-specific antibody-producing cells, comprising contacting said cells with said composition of claim 17.

35. An antiserum specific for a recombinant protein as claimed in claim 14.

36. A method for diagnosing an allergic reaction to pollens from both monocotyledenous and dicotyledenous plants, which comprises administering to an individual with the recombinant protein of claim 14, and evaluating a response to said administration.

37. A method for diagnosing an allergic reaction to pollens from both monocotyledenous and dicotyledenous plants, which comprising contacting serum from an individual with the recombinant protein of claim 14, and determining the formation of a complex between said recombinant protein and pollen-specific IgE antibodies present in said serum.

FIG.1A.

```
                10                  20                  30                  40                  50                  60
                *                   *                   *                   *                   *                   *
GAA TTC TTG CGC GAT GTT CTC GCC GCG CAG TGT CTT GAC CTT CTC GCC GTC GAT GAA CAC
    L   R   D   V   L   A   A   Q   C   L   D   L   L   A   V   D   E   H

                70                  80                  90                  100                 110                 120
                *                   *                   *                   *                   *                   *
CGG CGC GGC CGG ATT CTC TCC CGA CCC CGG CAA GCT GAT GCC GAT ATT CGC CTG CTT CGA
 R   R   G   R   I   L   S   R   P   R   Q   A   D   A   D   I   R   L   L   R

                130                 140 FORWARD         150                 160                 170                 180
                *                   * PRIMER SITE       *                   *                   *                   *
                                    →
CTC GCC CGG ACC GTT GAC GAT ACA GCC CAT CAC CGC CAC GTG CAT CTT CTC GAC GCC AGG
 L   A   R   T   V   D   D   T   A   H   H   R   H   V   H   L   L   D   A   R

                190                 200                 210                 220                 230                 240
                *                   *                   *                   *                   *                   *
ATA CTG ATC GCG CCA CAC CGG CAT CTG CGT ACG CAG ATA GGT CTG GAT TTG CGA CGC CAG
 I   L   I   A   P   H   R   H   L   R   T   Q   I   G   L   D   L   R   R   Q
```

FIG.1B.

```
            250         260         270         280         290         300
             *           *           *           *           *           *
TTC CTG GAA CAG TGT GCT GGT GGT GCG GCC ACA ACC CGG GCA CGC GAT CAC CAT CGG CGT
 F   L   E   Q   C   A   G   G   A   A   T   T   R   A   R   D   H   H   R   R

            310         320         330         340         350         360
             *           *           *           *           *           *
GAA CGA GCG CAG ACC CAT GGT CTG CAG GAT TTC CTG ACC GAC GAC CAC CTC GCC GGT GCG
 E   R   A   Q   T   H   G   L   Q   D   F   L   T   D   D   H   L   A   G   A

            370         380         390         400         410         420
             *           *           *           *           *           *
CGG GCC GCC CGG TTC CGG CGT CAG CGA GAT GCG GAT CGT GTC GCC GAT GCC CTG CTG CAG
 R   A   A   R   F   R   R   Q   R   D   A   D   R   V   A   D   A   L   L   Q

            430         440         450         460         470         480
             *           *           *           *           *           *
CAA TAC GGA CAA CGC AGC GGT TGA CGC CAC GAT GCC CTT GGA GCC CAT GCC GGC TTC GGT
 Q   Y   G   Q   R   S   G  STOP

REVERSE     490         500
PRIMER SITE  *           *
CAA GCC AAG ATG CAG CGC GAA TTC
```

SUBSTITUTE SHEET

2/5

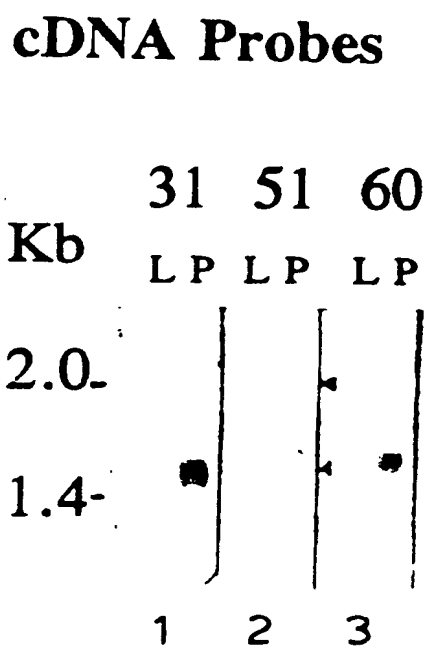

FIG.2.

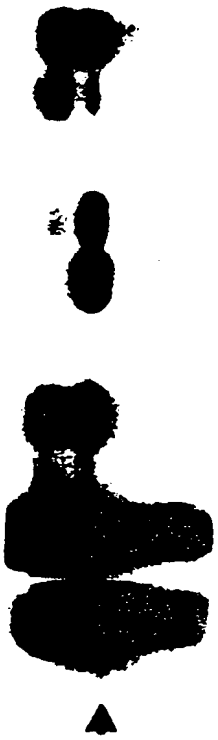

FIG.3.

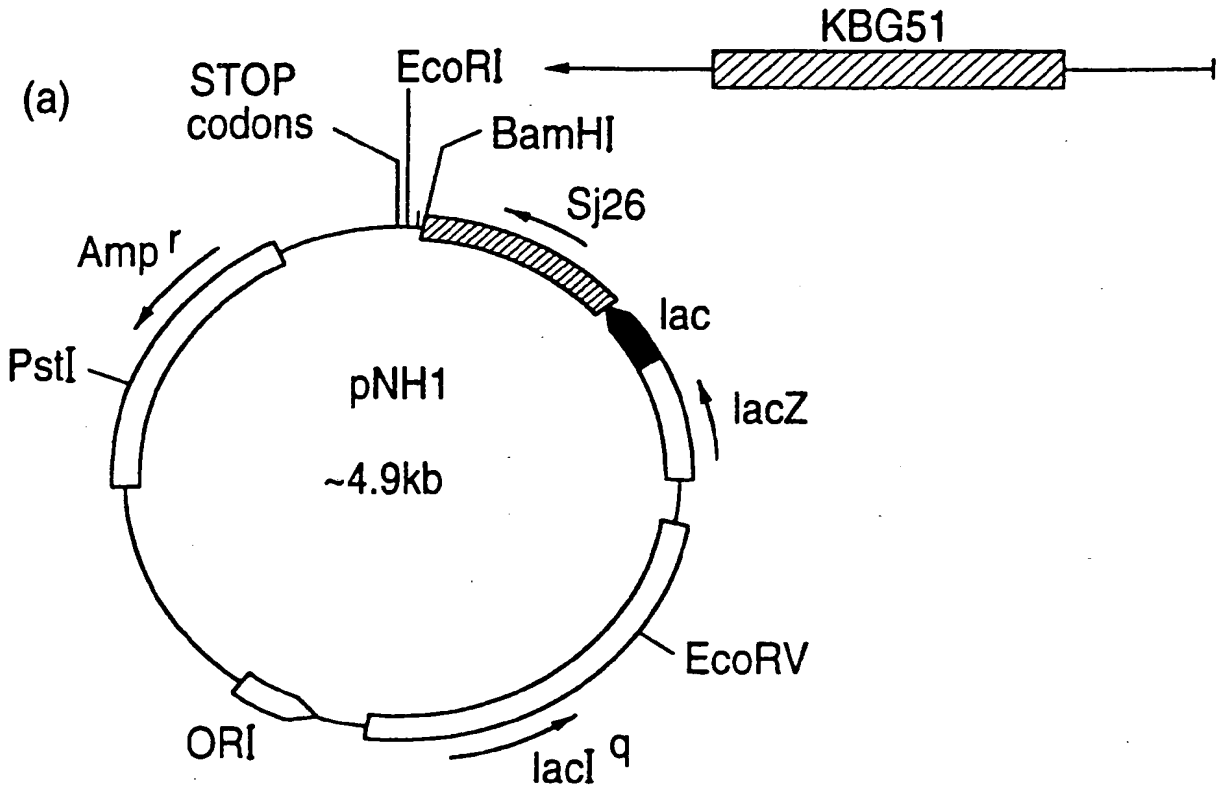

FIG.4.

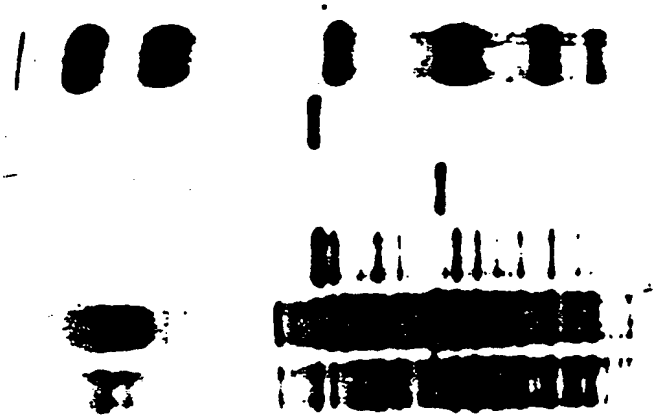

FIG. 5.

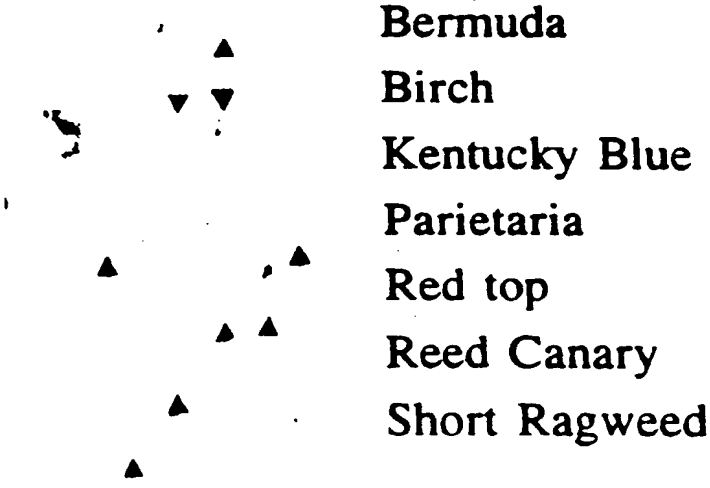

FIG. 6.

INTERNATIONAL SEARCH REPORT

Internatic ' Application No
PCT/LA [redacted]0021

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C07K14/415 A61K39/36 C07K16/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 03551 (BIOMAY BIOTECHNIK PRODUKTIONS-UND HANDELSGESELLSCHAFT M.B.H.) 5 March 1992 see page 22 - page 23, line 15 ---	1-6,34
X	CLINICAL AND EXPERIMENTAL ALLERGY, vol. 20,no. 5, August 1990 OXFORD, GB, pages 501-509, DONOVAN AND BALDO 'Crossreactivity of IgE antibodies from sera of subjects allergic to both ryegrass pollen and wheat endosperm proteins: evidence for common allergenic determinants' see the whole document --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 1995	24.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 93,no. 1, part 2, January 1994 page 231 LUO ET AL. 'Identification of a novel highly crossreactive allergen in Kentucky Bluegrass pollen' see Abstract 413 ---	1-6
A	JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 266,no. 2, 15 January 1991 MD US, pages 1204-1210, SILVANOVICH ET AL. 'Nucleotide sequence analysis of three cDNAs coding for Poa pIX isoallergens of Kemtucky Bluegrass pollen' see the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EA95/00021

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 29-33 and 36 are directed to methods of treatment and diagnostic methods practised on the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internat l Application No
PCT/CA 95/00021

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9203551	05-03-92	AT-A-	74094	15-02-95
		AU-A-	8390191	17-03-92
		CA-A-	2067182	14-02-92
		EP-A-	0495064	22-07-92
		JP-T-	5502589	13-05-93